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Correlation of Patient Immune Responses With Genetically Characterized Small Round-Structured Viruses Involved in Outbreaks of Nonbacterial Acute Gastroenteritis in the United States, 1990 to 1995

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Small round-structured viruses (SRSVs) are a genetically and antigenically diverse group of caliciviruses that are the most common cause of outbreaks of acute nonbacterial gastroenteritis. We have applied both molecular techniques to characterize SRSVs in fecal specimens and serologic assays using four different expressed SRSV antigens to examine the distribution of outbreak strains in the United States and determine if the immune responses of patients were strain specific. Strains from 23 outbreaks of SRSV gastroenteritis were characterized by reverse transcription-PCR and nucleotide sequencing of a 277-base region of the capsid gene. These strains segregated into two distinct genogroups, I and II, comprising four and six clusters of strains respectively, each representing a distinct phylogenetic lineage. Serum IgG responses in patients were measured by enzyme immunoassay using expressed capsid antigens of Norwalk virus (NV), Toronto virus (TV), Hawaii virus (HV), and Lordsdale virus (LV), representing four of the 10 clusters. While strains in genogroups I and II were antigenically distinct, within genogroups, the specificity of the immune response varied greatly. Patients infected with genogroup I strains which had as much as 38.5% aa divergence from NV demonstrated relatively homologous seroresponses to the single NV antigen. In contrast, in genogroup II, homologous seroresponses to TV and HV were only present when the infecting strains showed less than 6.5% aa divergence from these antigens. These results suggest that TV and HV represent not only separate genetic clusters in genogroup II but also separate antigenic groups, each of which is related but distinguishable. In addition, two genetically distinct SRSV strains were identified for which we have no homologous antigen. This study suggests that while current molecular diagnostics are capable of detecting the full range of SRSVs, additional expressed antigens will be required to detect an immune response to SRSV infection caused by all the antigenically diverse strains. ***J. Med. Virol. 53:372–383, 1997.***

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INTRODUCTION

Small round-structured viruses (SRSVs) are a group of genetically and antigenically diverse single-stranded (ss) RNA viruses of the family *Caliciviridae* [Jiang et al., 1990, 1993] that are the major cause of food- and waterborne outbreaks of acute nonbacterial gastroenteritis. The sequencing of the complete genomes of Norwalk virus (NV; Jiang et al., 1990, 1993) and Southampton virus [Lambden et al., 1993] enabled the characterization of additional strains and has facilitated phylogenetic analysis of the SRSVs. SRSVs can be differentiated into two distinct genogroups: genogroup I which includes NV, Southampton virus, and Desert Shield virus, and genogroup II which includes Toronto virus (TV), Hawaii virus (HV), Lordsdale virus (LV), Bristol virus (BV), Mexico virus (MV), Camberwell virus (CAV), Snow Mountain agent (SMA), and Melksham virus (MK) [Ando et al., 1994, 1995; Cauchi et al., 1996; Cubitt et al., 1994; Dingle et al., 1995; Green, SM et al., 1994, 1995; Lew et al., 1994a,b; Liu et al., 1995; Wang et al., 1994].

Comparison of these sequences has shown that the RNA polymerase region of ORF1 is the most conserved, while considerable variation exists between strains in both ORF2, encoding the capsid protein, and ORF3 which encodes a protein whose function remains undefined [Green, SM et al., 1994, 1995, 1997; Lew et al., 1994b; Wang et al., 1994]. For this reason, detection of SRSVs by reverse transcription-PCR (RT-PCR) has been mostly limited to the RNA polymerase region of the genome [Ando et al., 1994, 1995; Green, J et al., 1993; Moe et al., 1994; Norcott et al., 1994; Wolfaardt et al., 1995]. Variation, even in an 81-base nucleotide sequence of the RNA polymerase, was sufficient to enable us to differentiate five groups of genetically distinct SRSVs, which corresponded to four of the antigenic types determined by using solid phase IEM (SPIEM) [Ando et al., 1995]. These genetic groups most closely correspond to those represented in Genbank by NV (P1-A, UK2), TV (P2-A, UK1), HV and LV (P2-B, UK3/UK4), and P1-B (UK1) a strain for which there is no Genbank equivalent.

The development of specific and sensitive enzyme immunoassays (EIAs) using baculovirus-expressed recombinant capsid proteins of NV (rNV) [Jiang et al., 1990, 1992, 1993], TV (rTV) [Leite et al., 1996], Mexico virus [Jiang et al., 1995], HV (rHV) [Green, KY et al., 1997] and LV (rLV) [Dingle et al., 1995] has allowed researchers to detect differences in the seroresponses of patients to infections with genetically distinct strains [Monroe et al., 1993; Parker et al., 1993, 1995]. While these strains have been shown to be both genetically and antigenically distinct, the diversity of genetically characterized SRSV strains causing outbreaks in the community has not been correlated with the results obtained by serologic testing using the recombinant-expressed antigens.

In this study, we examined the genetic and antigenic diversity of 23 SRSV strains present in specimens collected during outbreaks of gastroenteritis that were investigated by the Centers for Disease Control and Prevention (CDC), in an effort to correlate the genetic differences observed in the infecting strains with patient seroresponses to the infection. We used nucleotide sequences of RT-PCR products derived from two regions of the SRSV genome, an 81-base region of the RNA polymerase gene and a 277-base region located toward the 5′-end of ORF2, to genetically characterize the outbreak strains. We then measured patient seroresponses to a panel of four genetically and antigenically distinct baculovirus-expressed SRSV capsid proteins. In particular, we wanted to determine whether patients exhibit specific immune responses to the expressed capsid proteins when the outbreak strain is of the same genetic lineage i.e., if there is a direct correlation between the strains that were genetically similar to NV, TV, HV, and LV and the seroresponses of these patients to the rNV, rTV, rHV, and rLV antigens, respectively. These efforts should provide information, not only on the diversity of strains causing outbreaks in the US, but also facilitate identification of those reagents most critical for use in routine diagnosis of SRSV infections.

MATERIALS AND METHODS

Outbreaks/Specimens

The 23 SRSV outbreaks selected for use in this study were from a collection of 43 outbreaks caused by SRSV strains that had been genetically characterized by RT-PCR, Southern hybridization, and nucleotide sequence analysis in the RNA polymerase region of the genome according to the method of Ando et al. [1995]. These outbreaks represent a subset of a national collection of 67 outbreaks of SRSV gastroenteritis that occurred in the United States and were reported to the CDC between 1990 and 1995. The outbreaks were selected as being representative of the genetic diversity of SRSV strains encountered, for which both stool and serum specimens were available. One additional outbreak caused by Parkville virus, a strain related to Sapporo virus and recently described as a Group C calicivirus [Noel et al., 1997], was included in the study collection as a negative control. The outbreaks are identified by a code indicating the CDC outbreak number, the CDC specimen identification number of the SRSV strain that was sequenced, the year and the state in which the outbreak occurred (Table I). The outbreaks, from 13 states in the United States, occurred in a variety of settings including nursing homes, schools, restaurants and cruise ships among people ranging in age from young children to the elderly. In general, clinical specimens were submitted to the CDC according to the published guidelines requiring that stool specimens be collected within 48 to 72 hours and acute- and convalescent-phase sera be collected 1 to 7 and 21 to 42 days, respectively, following onset of diarrhea [Lew et al., 1990]. Stool specimens were stored at 4°C and sera at −20°C.

TABLE I. SRSV Outbreak Strains Used in This Study

Identification[a]	Outbreak setting, mode of transmission (if known)	Mean age of patients (range)
184/01388/90/CS	Cruise ship	na[b]
201/00M2/91/NY	Tennis meet, foodborne	33 (21–56)
202/01815/91/WA	Nursing home	na
247/02553/93/PA	University	20 (17–23)
262/02803/93/WA	Park/camp, ?lakewater	30 (<1–46)
273/11860/94/PA	College, foodborne	39 (18–73)
277/11873/94/MD	Restaurant	37 (25–56)
279/11885/94/LA	na,[b] catered meal	39 (19–57)
283/12039/94/VA	Nursing home	94 (83–100)
290/12275/94/VT	Hospital	57 (31–82)
292/11641/94/HI	Cruise ship	na
293/12359/94/VA	Nursing home	86 (79–101)
295/12401/94/MO	Nursing home	81 (71–99)
297/12575/94/KS	Track meet, implicated ice	36 (16–51)
299/00000/94/MD	School, PTA luncheon	43 (25–60)
301/12630/94/CS	Cruise ship	16 (na)
302/12678/94/MD	Nursing home[c]	84 (37–101)
304/34839/94/KS	Wedding, catered meal	na
306/12732/94/LA	Wedding, catered meal	na
312/08377/94/AL	School	40 (10–51)
313/08384/94/MD	Company, catered meal	41 (30–61)
314/08410/94/HI	Hotel, cocktail party	43 (26–60)
315/08432/94/MA	University, cafeteria[d]	18 (16–28)
316/08438/94/AR	School, teacher luncheon	37 (8–51)

[a]CDC outbreak number/CDC specimen identification number of the specimen that was sequenced/year of the outbreak/US state in which the outbreak occurred. CS, Cruise ship.
[b]na, data not available.
[c]Rodriguez et al., 1996.
[d]Kilgore et al., 1996.

Capsid Primers

A similarly plot of genogroup II SRSV ORF2 sequences from Genbank was used to design primers, mon381, and mon383 (Table II). These primers, directed toward two relatively conserved regions near the 5′-end of ORF2 were used to amplify a 322bp region of the capsid gene of the majority of genogroup II strains. Additional primer pairs, jsn3 through jsn10, were designed using partial nucleotide sequence data of cloned SRSV strains from outbreaks 277, 306, 314, and 316 [Seto et al., manuscript in preparation] to amplify two genogroup I and two genetically distinct genogroup II strains. These primers, amplified 418bp, 400bp, 389bp, and 354bp fragments respectively, overlapping the region corresponding to that amplified by mon381 and mon383.

Genetic Characterization

SRSV strains from at least one stool specimen from each outbreak were analyzed in the capsid region by RT-PCR according to the method of Ando et al. [1995a], using modified thermal cycling conditions of 72°C for extension in the PCR step. Nucleotide sequencing of both strands of the amplicons were performed using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Foster City, CA) on an automated sequencer (Applied Biosystems model 377, Applied Biosystems Inc, Foster City, CA).

Phylogenetic analysis of sequence data was performed using PAUP [Swofford, 1991] and the GCG suite of programs [Genetics Computer Group, 1994]. A phylogram of the RNA polymerase region from the aligned 81-(unique) base nucleotide sequences of the strains in our collection of 43 characterized outbreaks, 20 previously characterized UK strains [Ando et al., 1995], and 13 strains from Genbank, used to select the outbreaks for this study, was compared with a phylogram generated from the aligned 277-(unique) base nucleotide sequences of the capsid region of 20 of the 23 selected outbreak strains, eight UK strains and 13 strains from Genbank. The 23 outbreak strains were genetically categorized on the basis of their predicted phylogenetic relatedness in the capsid region to NV, TV, HV, and LV, the strains for which we have baculovirus-expressed capsid proteins available. Strains which could not be amplified in the capsid region were categorized on the basis of their predicted phylogenetic relatedness to NV, TV, HV, and LV in the RNA polymerase region. For our study, a cluster was defined as two or more closely related strains arising from a single genetic lineage. Thus, all strains which genetically were closely related to NV were categorized into the NV-cluster and those that were related to TV, HV, and LV were categorized into the TV-, HV-, and LV-clusters respectively.

As the majority of these sequences represent fragments of the entire capsid sequences which will be available in a separate paper [Seto et al., manuscript in preparation], they have not been deposited in Genbank. GenBank accession numbers for strains used in the analysis are: Bristol virus, X76716; Camberwell virus, U46500; Desert Shield virus, U04469; Hawaii virus, U07611; KY89, L23828; Lordsdale virus, X86557; Melksham virus, X81879; Mexico virus, U22498; Norwalk virus, M87611; OTH25, L23830; Snow Mountain agent, L23831; 475682 Southampton virus, L07418; and Toronto virus, U02030.

Serologic Characterization

Serum immunoglobulin G (IgG) responses to NV, TV, HV, and LV of patients from the 24 outbreaks were measured by direct EIA using the recombinant-expressed capsid proteins as antigens (rNV-EIA, rTV-EIA, rHV-EIA, and rLV-EIA, respectively). The method for the rTV-, rHV-, and rLV-EIA's was adapted from procedures previously described for the rNV-EIA [Monroe et al., 1993]; the rNV-EIA was performed as described. Levels of IgG were determined at a single test dilution of patients' sera; 1 in 500 for the rNV-EIA and 1 in 1,000 for the recently developed rTV-, rHV- and rLV-EIA's. Briefly, positive antigen wells of Immulon II plates (Dynatech, Chantilly, VA) were coated with 1.1 μg of rTV per ml, 1.0 μg of rHV per ml or 1.0 μg of rLV per ml, and negative antigen wells were coated with an equivalent concentration of purified AcNPV (baculovirus wild type) at 4°C overnight. The plates were blocked for 1 hr at 37°C with 5% BLOTTO, washed, then 75 μl of patient sera and reference sera

TABLE II. Description of Oligonucleotide Primers

Geno group	Primer	Sequence (5′ to 3′)	Polarity	Location[a]	Ref[b] strain
I	jsn3	ccc agg gcg agt tta caa	+		277
I	jsn4	acc aca aaa gaa tca gtc cca	−		277
I	jsn5	tat cgc cta aca ata cac cag	+		316
I	jsn6	aat cac aaa agg tca gtt cca	−		316
II	mon381	cca gaa tgt aca atg gtt atg c	+	5362–5383	LV
II	mon383	caa gag act gtg aag aca tca tc	−	5661–5683	LV
II	jsn7	aga aat tcc cct gga gag ata	+		306
II	jsn8	cgg tga aaa aac gtc gtc	−		306
II	jsn9	ccc cga act aaa tcc ata c	+		314
II	jsn10	cag gag aca gta aat aca tca tc	−		314

[a]Location refers to the equivalent nucleotide location within the Lordsdale virus genome (X86557). All other primers were designed using partial sequence information from cloned SRSV strains.
[b]LV, Lordsdale virus, other numbers refer to the outbreak number of the cloned strain used to design the primers.

was added to the wells and incubated for 3 hr at 37°C. The TV reference serum (CDC 91-1821) was obtained from a patient involved in a P2-A type outbreak (strain number 1 in Table I of reference Ando et al., 1995; and Table I, outbreak 202). The HV (CDC 94012705, Table I, outbreak 302) and the LV (CDC 95011990) reference sera were obtained from patients involved in genogroup II, P2-B type outbreaks. The reference sera were selected as they showed strong seroresponses to TV, HV and LV antigens, respectively, although some cross reactivity against the other antigens was observed for the TV and HV reference sera. The plates were washed, 75 μl of a 1:50,000 dilution of peroxidase-conjugated goat anti-human IgG (GIBCO BRL, Grand Island, NY) was added, and the plates were incubated for 1 hr at 37°C. Bound IgG was detected by using 125 μl of a 5 mg solution of 3,3′,5,5′-tetramethyl benzidine (Sigma Chemical Co., St. Louis, MO) per ml, per well. The reaction was stopped after 15 min with 50 μl of 2N H_2SO_4 per well, and the A_{450} was read with a microplate spectrophotometer (MR5000; Dynatech). The IgG units of individual patient sera against each antigen were calculated using the 5 to 90% range of a standard curve generated from the serial titrations of the reference sera on each test plate. Seroconversion was defined as a fourfold or greater rise in antibody units between the acute- and convalescent-phase sera from an individual patient.

RESULTS

Analysis of the RNA Polymerase Sequences

The dendrogram based on the 81-base nucleotide sequence from a total of 77 strains clearly indicated the two phylogenetically distinct lineages comprising genogroups I and II (Fig. 1). Within each genogroup, strains could be differentiated into many phylogenetic branches on the basis of their nucleotide sequences. For genogroup I, 16 strains including NV, formed clusters along multiple lineages. We had previously placed the majority of these strains in the P1-A genetic group [Ando et al., 1995]. Segregated from these 16 strains was a cluster of five closely related strains corresponding to what we had previously called the P1-B genetic group.

The greater number of strains examined in this study allowed us to identify phylogenetic relationships that had been unclear previously. Earlier we differentiated genogroup II strains into two groups, P2-A which included TV and MV and P2-B which included HV, LV, and SMA. However the P2-B group did not correlate directly with the UK3 and UK4 antigenic types of these strains [Ando et al., 1995]. In this study, and consistent with our previous results for the P2-A group, 16 of 56 strains formed a cluster with TV, distinct from the remaining clusters of genogroup II strains. However, the strains within the P2-B group which previously appeared to form a single cluster with LV, BV, SMA, and HV, can now clearly be differentiated into at least two lineages, one with 23 strains surrounding LV and another with 10 strains surrounding HV. Of note, SMA, the previously reported prototype strain of genogroup II [Wang et al., 1994], was placed in a lineage distinct from the other genogroup II strains.

Analysis of the Capsid Sequences

Nucleotide sequences of a 277 base region of the 20 strains we were able to amplify in the capsid region were compared with the sequences of 8 strains antigenically characterized as UK1, UK3, or UK4 [Ando et al., 1995] and 13 strains from Genbank (Fig. 2). Overall, in the dendrogram based on the capsid sequence, the segregation of strains into two distinct genogroups each with multiple clusters was consistent with the genogroups and clusters identified in the dendrogram based on the RNA polymerase region. For genogroup I strains, the multiple clusters represented by the P1-A strains in the RNA polymerase could be differentiated into at least 4 clusters in the capsid region represented by NV, SOV, DSV, and CS (Cruise Ship, where outbreak 184 occured). However, the cluster represented by the strain from outbreak 292 and the previously characterized strains, UK1-6 and UK1-7, placed in genogroup I on the basis of the polymerase analysis,

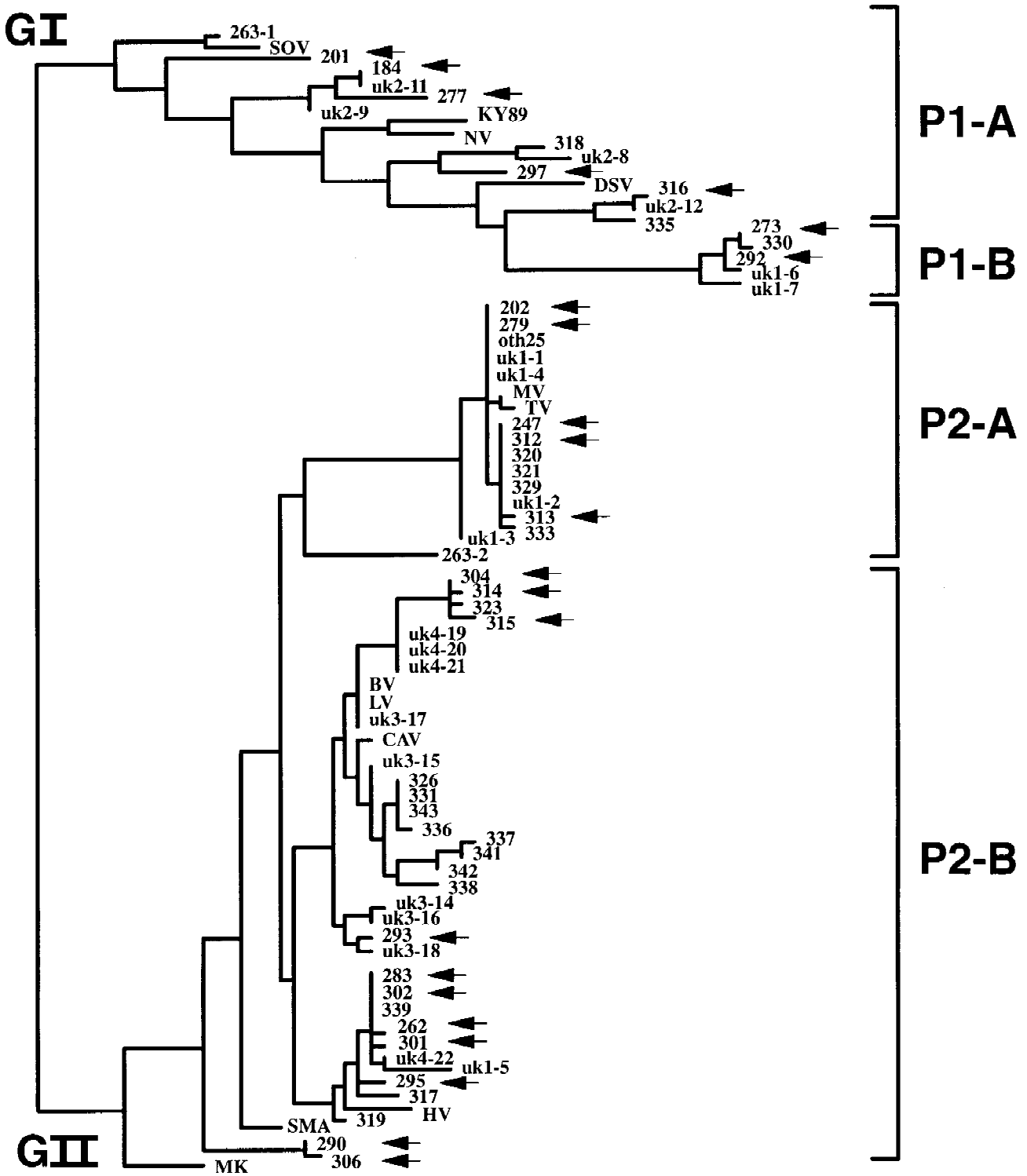

Fig. 1. One of many equally parsimonious trees generated by PAUP with a 5:1 weighting of transversions to transitions, using the 81-base nucleotide sequences from the RNA polymerase region of SRSV outbreak strains. Nucleotide sequences included are those of 43 outbreak strains, 20 previously described UK strains [Ando et al., 1995] and 13 strains from GenBank. Strains from outbreaks examined in this study are indicated by arrows. Abbreviations and GenBank accession numbers for strains are: GI, Genogroup I; GII, Genogroup II; SOV, Southampton virus, L07418; KY89, L23828; NV, Norwalk virus, M87611; DSV, Desert Shield virus, U04469; OTH25, L23830; MV, Mexico virus, U22498; TV, Toronto virus, U02030; BV, Bristol virus, X76716; LV, Lordsdale virus, X86557; CAV, Camberwell virus, U46500; HV, Hawaii virus, U07611; SMA, Snow Mountain agent, L23831 475682 and MK, Melksham virus, X81879.

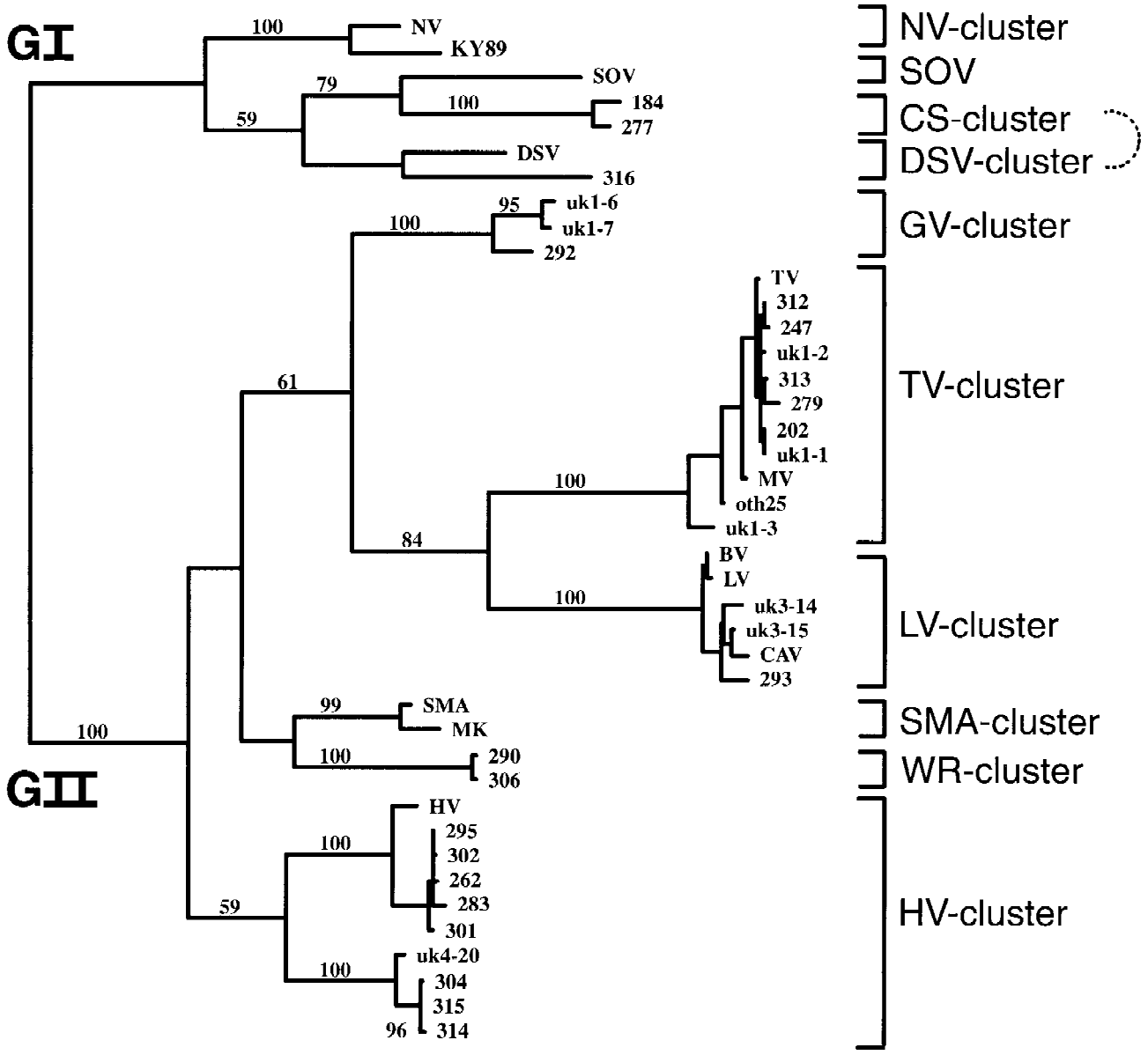

Fig. 2. The single most parsimonious tree generated by PAUP with a 5:1 weighting of transversions to transitions, using the 277-base nucleotide sequences from the capsid region of 20/23 SRSV outbreak strains, eight UK strains and 13 strains from Genbank. The results of the genetic differentiation of strains into clusters on the basis of nucleotide and amino acid identities are indicated together with boot strap values from 100 replicates. The dotted line indicates two genogroup I clusters represented in our collection where more than 50% of patients seroresponded to the rNV antigen. Strain abbreviations are as described for Figure 1. CS, Cruise ship cluster, GV, Gwynedd Virus cluster, WR, White River cluster, named for the location where the first outbreak in these clusters occurred.

was placed in genogroup II on the basis of the capsid analysis. This cluster was named the Gwynedd virus (GV) cluster after the location where outbreak 273 occurred. For genogroup II strains, the three clusters represented by TV, LV and HV in the dendrogram based on the RNA polymerase sequence were more clearly defined in the dendrogram based on the capsid sequence. Of note, four strains from outbreaks 304 315, 314 and the strain UK4-20 shifted from a lineage within the LV-cluster based on the polymerase gene to a lineage related to HV based on the capsid sequence.

The phylogenetic relationships among the 41 strains predicted on the basis of the nucleic acid sequence (including the suggested four clusters in genogroup I and the four clusters represented by GV, TV, LV, and HV strains in genogroup II) were more clearly defined by analysis of the deduced amino acid sequences. Pairwise comparison of the alignments of the amino acid sequences indicated identities of 93.4 to 100% within individual clusters, 61.5 to 90.2% between clusters within a genogroup and 40.7 to 58.7% between genogroups I and II (Table III). An amino acid identity of 95.7% between HV and the four strains from outbreaks 304 315, 314 and UK4-20 (which exhibited between

TABLE III. Ranges of Uncorrected Percent Nucleotide (nt) and Amino Acid (aa) Identities of Strains Between Clusters Using 277 Bases of the Capsid Region

A. Genogroup I strains

	DSV-cluster	NV-cluster	CS-cluster	SOV-cluster	
DSV-cluster	79.2 93.4	62.8–65.0	66.8–71.9	67.9–71.9	
NV-cluster	61.5–64.8	89.2 96.7	64.2–64.6	62.4–62.8	
CS-cluster	71.4–76.9	64.8–65.9	92.3 97.8	73.4–74.1	nt
SOV-cluster	72.5–75.8	67.0	80.2–82.4	100.0 100.0	
		aa			

B. Genogroup II strains

	TV-cluster	HV-cluster	LV-cluster	GV-cluster	WR-cluster	SMA-cluster	
TV-cluster	89.9–100.0 96.7–100.0	66.1–71.8	69.0–71.8	66.4–69.0	65.0–67.9	63.5–68.2	
HV-cluster	70.7–76.1	78.4–99.6 93.5–100.0	68.6–74.0	69.3–74.0	70.0–76.5	74.7–80.1	
LV-cluster	75.0–78.3	70.7–73.9	93.1–98.9 94.6–100.0	63.9–67.9	66.8–69.0	66.8–70.4	nt
GV-cluster	75.0–78.0	76.1–79.1	69.6–73.6	89.1–95.6 98.9–100	67.2–68.2	70.1–73.3	
WR-cluster	68.5–71.7	80.4–83.7	68.5–72.8	73.9–76.9	97.5 98.9	75.5–77.6	
SMA-cluster	69.6–72.8	85.9–90.2	70.7–75.0	76.1–79.1	85.9–88.0	93.5 97.8	
			aa				

79.4% and 84.1% nucleotide identity to other members of the HV-cluster) allowed us to group them in the HV cluster. In addition, the amino acid analysis suggested two additional clusters: one includes strains 290 and 306 which was named the White River (WR) cluster after the location of the first outbreak and the other includes strains SMA and MK.

Serologic Characterization

Using our collection of 23 outbreaks caused by strains characterized on the basis of the capsid region into four clusters (NV, SOV, CS, and DSV) within genogroup I and 6 clusters (GV, TV, LV, SMA, WR, and HV) within genogroup II, and the specimens from the Parkville virus outbreak, we measured patient IgG seroresponses to our panel of four baculovirus-expressed capsid proteins (Table IV). Consistent with the genetic differences between genogroup I and II strains, we observed very little cross-reaction in patient seroresponses to the antigens between genogroups. Although genetically, our collection of genogroup I strains formed 4 distinct clusters, the seroresponses (with the exception of those from outbreak 201) to the rNV antigen of patients infected with these strains showing up to 38.5% amino acid divergence from NV indicated that antigenically they are related to NV. Thus, for subsequent serologic comparisons, all genogroup I outbreak strains in our collection will be referred to as belonging to the NV-cluster. In contrast, for our collection of genogroup II strains, analysis of antibody rises by cluster

TABLE IV. Genetic and Immunogenic Characterization of Outbreak Specimens[a]

Geno-group	Cluster	Identification	Stools (N)	RT-PCR pol[b] Positive (%)	RT-PCR cap[c] primers	Sera (N)pr	Seroconversions rNV[d]	rTV[d]	rHV[d]	rLV[d]
I	CS	184	1	1[e]	jsn3/4	12	**7**	0	0	0
		277	13	8 (62)	clone[f]	10	**7**	0	0	0
I	DSV	316	12	4 (33)	jsn5/6	6	**5**	0	0	0
I	?	201	5	3 (60)	na[g]	5	2	0	0	2
I	?	297	8	3 (38)	na[g]	7	**7**	0	1	0
		Totals	39	19 (49)		40	70%	0%	3%	5%
II	TV	202	1	1[h]	mon381/383	4	1	**4**	2	1
		247	7	7 (100)	mon381/383	5	0	**4**	1	0
		279	1	1 (100)	mon381/383	12	2	**8**	**7**	1
		312	6	5 (83)	mon381/383	20	0	8	6	4
		313	11	8 (73)	mon381/383	9	2	**8**	**7**	2
		Totals	26	22 (85)		50	10%	64%	46%	16%
II	HV	262	5	1 (20)	mon381/383	12	0	**5**	**6**	0
		283	11	8 (73)	mon381/383	6	1	3	**6**	2
		295	8	5 (63)	mon381/383	6	1	2	**5**	1
		301	6	5 (83)	mon381/383	17	2	**9**	**10**	2
		302	18	10 (56)	mon381/383	15	3	3	**13**	4
		304	2	1 (50)	jsn9/10	10	0	0	0	0
		314	3	2 (67)	clone[f]	10	1	4	3	2
		315	9	5 (56)	mon381/383	12	3	4	**10**	4
		Totals	62	37 (60)		88	13%	34%	57%	17%
II	LV	293	10	8 (80)	mon381/383	10	1	5	**6**	**6**
		Totals	10	8 (80)		10	10%	50%	60%	60%
II	GV	273	4	1 (25)	na[g]	16	1	1	5	2
		292	2	1 (50)	mon381/383	14	0	1	0	1
		Totals	6	2 (33)		30	3%	6%	17%	10%
II	WR	290	14	6 (43)	jsn7/8	7	1	2	3	0
		306	7	4 (57)	clone[f]	8	3	**4**	**4**	2
		Totals	21	10 (48)		15	27%	40%	47%	13%
Parkville[i]		299	7	0	—	14	0	0	0	0

[a]Outlined areas highlight the serologic results obtained using the homologous antigen to the genetic cluster of the outbreak. Bold type indicates where greater than 50% of outbreak patients showed greater than 4-fold rises to the expressed antigen [Monroe et al., 1993].
[b]pol, Polymerase.
[c]cap, Capsid.
[d]rNV/rTV/rHV, baculovirus expressed recombinant Norwalk virus/Toronto virus/Lordsdale virus/Hawaii virus capsid proteins.
[e]From Ando et al., 1995, Strain number 11, only one specimen was tested.
[f]Seto et al., manuscript in preparation.
[g]na, Data not available, strain could not be amplified in the capsid region.
[h]From Ando et al., 1995, Strain number 1, only one specimen was tested.
[i]Parkville virus, from Noel et al., 1997.

showed that the greatest number of responses to the rTV or rHV were among patients involved in an outbreak of the same cluster. Individually, some patients seroresponded to two, three, or all four of the antigens, particularly patients from the one LV-cluster outbreak and the two WR-cluster outbreaks who demonstrated higher levels of cross-reactivity among the three genogroup II antigens although fewer specimens from these clusters were available for testing than for both TV and HV. For the GV-cluster, only a few patients seroresponded to any of the four antigens; these strains appear to induce an antigenically distinct seroresponse in patients. Of note, the serologic results for outbreak 304 suggest that the SRSVs detected in one of two stool specimens available from the outbreak (neither of which were from the same patients as any of the serum pairs) may not have been the true cause of the outbreak and is excluded from further analysis. No patients involved in our control outbreak (Parkville-299) seroconverted to any of the four antigens.

Analysis of the frequency and magnitude of antibody rises by genetic cluster demonstrated that responses to the expressed antigens were the most common and strongest among patients involved in an outbreak caused by a strain of the same cluster (Fig. 3). For example, the frequency of seroconversions within an individual NV-cluster outbreak ranged from 40–100% (mean 70%), with an average rise of 15.4-fold (range 0.1 to 131.0) to the rNV antigen, whereas the frequency of seroconversions to the rTV, rHV, and rLV antigens ranged from 0–40% (mean 4.5%) with rises of 1.2- to 1.6-fold (range 0.6 to 11.1). Similarly, the frequency of seroconversions within individual TV- or HV-cluster outbreaks ranged from 40–100% (mean 75%) and 30–100% (mean 70%) with average rises of 21.0-fold (range 0.7 to 145.1) and 21.3-fold (range 0.7 to 773.5) to the rTV and rHV antigens, respectively. Although some cross-reactions were observed within genogroup II strains, the frequency and magnitude of antibody rises observed in the sera from these patients to the heter-

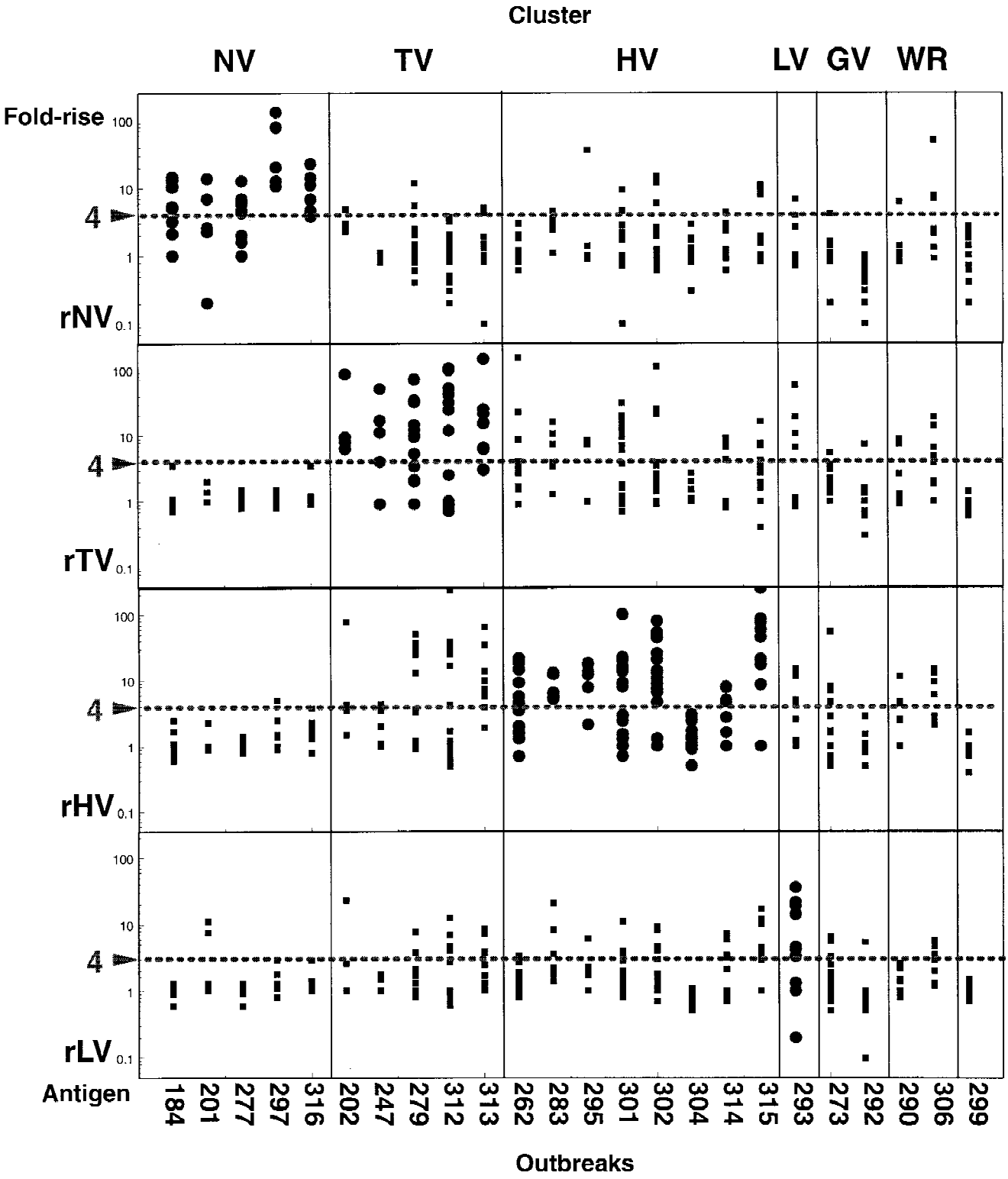

Fig. 3. Graphs of the magnitude of antibody rises to rNV, rTV, rHV, and rLV by genetic cluster. Outbreaks according to Table III are represented along the abscissa, magnitude of antibody rises along the ordinate axis. Antibody rises to the homologous antigen are indicated by circles, and by squares to the heterologous antigens. The dashed line represents a fourfold rise in antibody units.

ologous antigens were uniformly less than those to the homologous antigens.

Analysis of the magnitude of antibody rise and IgG levels in acute-phase serum of patients showed overall higher acute-phase titers to the rNV and rLV antigens than the rTV and rHV antigens (data not shown). These may reflect the historic predominance of NV- and LV-related strains and the lower acute-phase titers observed to the rTV and rHV antigens, may be related to the current predominance of TV- and HV-related strains.

DISCUSSION

From a collection of specimens from 24 outbreaks of gastroenteritis which occurred in 14 states in the United States, we have characterized the SRSV strain detected in stool specimens, measured the patients' immune response to infection, and examined the correlation between the nucleic acid sequence of the infecting strain in two regions of the genome and the seroresponse mounted by the patients. This study was possible through the application of two novel methods: RT-PCR, Southern hybridization, and nucleotide sequencing to detect and differentiate SRSV strains in stool specimens, and EIAs using four antigenically distinct baculovirus-expressed SRSV capsid proteins to measure the IgG antibody responses in paired patient sera.

In general, our data demonstrated a good correlation of phylogenetic lineages between the capsid and RNA polymerase regions. Genogroups I and II remained clearly defined in both ORFs. Our analysis of both the nucleotide and deduced amino acid sequences in the capsid region has allowed us to more clearly differentiate the clusters of strains suggested by phylogenetic analysis of the RNA polymerase region. One exception was the shift in lineage of the GV-cluster (P1-B) from genogroup I based upon sequence data in the polymerase region to genogroup II based upon sequence data in the capsid region. The capsid result is consistent with our amplification of these strains using the G-2 primer set [Ando et al., 1995] while our previous result based on the polymerase region may have been an artifact of using a short 81-base segment for the analysis.

Our 277-base fragment comprising about 17% of ORF2, has allowed us to draw some conclusions about overall capsid sequence diversity and immunogenicity of SRSV strains, particularly those belonging to the NV-, TV-, and HV-clusters which were well represented in our collection. This is despite amplifying a region of the capsid protein located toward the N-terminus, not the C-terminus to which monoclonal antibody binding sites have been mapped [Hardy et al., 1996] or the central hypervariable region [Green, SM et al., 1995] of ORF2. Additionally, while RT-PCR in the polymerase region demonstrated broad reactivity among different SRSV strains, RT-PCR in the capsid region was more difficult because of great sequence diversity in ORF2, requiring seven primer pairs to amplify 17 of the 23 SRSV outbreak strains. Sequence data for three additional strains was obtained only after cloning, of entire capsid region.

Our results are consistent with other studies demonstrating that strains belonging to genogroups I and II are not only genetically distinct [Green, SM et al., 1994] but also antigenically distinct [Lew et al., 1994b; Wang et al., 1994]. We observed very little cross-reaction in patient seroresponses to the expressed antigens between the two genogroups. However the seroresponses to the expressed antigens between patients infected with genogroup I strains were different to those infected with genogroup II strains. Despite our differentiation of genogroup I strains into four clusters (NV-, SOV-, CS, and DSV), patients infected with these strains (showing up to 37.6% nt and 38.5% aa divergence from NV) demonstrated relatively homogeneous seroresponses to the single rNV antigen. This contrasted with the seroresponses detected in patients infected with genogroup II strains. For example, patients infected with strains belonging to the TV- and HV-clusters exhibited good seroresponses only when the infecting strains showed less than 22.0% nt and 6.5% aa divergence from the respective antigens. For the remaining three clusters (LV, GV, and WR), small sample size and/or lack of related antigens precludes us from drawing firm conclusions. Further analysis with additional LV-cluster strains will be required to determine whether patients exhibit specific seroresponses to this antigen. Similarly, an expressed antigen related to the WR- and GV-clusters will be required to examine the specific immune responses in patients infected with these strains. The cross reactive seroresponses in outbreak patients to the three genogroup II antigens suggest that although, we were able to detect genetic differences between these strains, antigenically, these strains are related. Our observation that some patients seroconverted to one or more of the baculovirus-expressed antigens independent of the genetic type of the outbreak strain may be related to suggestions that IgG antibody responses to SRSVs are less type-specific than IgM antibody responses [Cubitt et al., 1987; Lew et al., 1994a; Lewis et al., 1988; Treanor et al., 1993].

Our examination of the genetic and antigenic diversity of a collection of SRSV strains and the correlation between the results of genetic characterization and measurement of immune response to antigenic types provides us with clear guidelines for future work in the study of SRSV gastroenteritis. Nine years ago, a review of 100 outbreaks of acute nonbacterial gastroenteritis examined by serologic testing for NV determined that only 25% of these outbreaks were attributable to NV infection whereas 42% were attributed to "Norwalk-like" variant strains on the crude basis that fewer than one half of patients seroconverted to the rNV antigen [Glass et al., 1989]. In characterizing the distribution of strains causing 43 outbreaks in the US between 1990 and 1995, in our collection viruses in the NV-cluster (genogroup I) have been relatively uncommon (8143) whereas genogroup II strains appear to be predomi-

nant. The genetic homogeneity observed within clusters of genogroup II strains is suggestive of their recent divergence from a single ancestor, a factor that may be related to their current predominance. An important consequence of this finding is that in the past, serologic studies using the NV antigen alone may have greatly underestimated the importance of this antigenically diverse group of viruses and in particular, overlooked genogroup II strains. Our observations that patient seroresponses to each cluster appear to be relatively specific for the homologous antigen using our antigen EIA and that, in our collection of 23 outbreaks up to six immunogenically distinct clusters are represented, suggests that while current molecular diagnostics are capable of detecting the full range of SRSVs, additional expressed antigens will be required to detect an immune response to SRSV infection caused by all the antigenically diverse strains. Of note, the expression of Parkville virus in baculovirus may provide us with similar results for this genetically and antigenically distinct group of the Caliciviridae.

While our current method of RT-PCR in the polymerase region can detect strains well, it is restricted in its ability to differentiate SRSV strains. RT-PCR in the capsid region has enhanced our ability to differentiate strains, but is limited by the greater sequence diversity in ORF2. An assay that is both simpler and more sensitive will be needed for the detection and differentiation of SRSVs in routine diagnostic specimens. While we have demonstrated a correlation between a patient's IgG seroresponse and the genetic type of the infecting strain, the measurement of patient IgM seroresponses may prove to be more specific. The distinguishing feature of these novel methods to detect SRSV infections by resting stool specimens and seroresponses in patients serum is that neither relies on nonreplenishable or human reagents, factors that have limited earlier attempts to study genetic and antigenic relationships between strains. Our data indicate that genetically similar strains are in circulation worldwide and that improved diagnostics will provide important tools for future studies of the epidemiology and disease burden caused by SRSV infection.

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